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REGULAR PAPER

Temporal variation in leaf nitrogen partitioning of a broad-leaved evergreen tree, *Quercus myrsinaefolia*

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Abstract We examined temporal changes in the amount of nitrogenous compounds in leaves from the outer and inner parts of the crown of *Quercus myrsinaefolia* growing in a seasonal climate. Throughout the leaf life span, metabolic protein and Rubisco content closely correlated with total nitrogen content, while structural protein content was relatively stable after full leaf expansion. Chlorophyll content was affected by shading as well as total nitrogen content in outer leaves that were overtopped by new shoots in the second year. Outer leaves showed a large seasonal variation in photosynthetic nitrogen-use efficiency (PNUE; the light-saturated photosynthetic rate per unit leaf nitrogen content) during the first year of their life, with PNUE decreasing from the peak in summer towards winter. Outer and inner leaves both showed age-related decline in PNUE in the second year. There were no such drastic changes in leaf nitrogen partitioning that could explain seasonal and yearly variations in PNUE. Nitrogen resorption occurred in overwintering leaves in spring. Metabolic protein explained the majority of nitrogen being resorbed, whereas structural protein, which was low in degradability, contributed little to nitrogen resorption.

Keywords Leaf ageing · Leaf senescence · Metabolic protein · Nitrogen resorption · Rubisco · Structural protein

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Introduction

Photosynthetic capacity (defined as "the light-saturated photosynthetic rate" in this study) generally shows good correlations with total leaf nitrogen content in many species (Field and Mooney 1986). In evergreens, however, temporal changes in photosynthetic capacity do not always mirror those in leaf nitrogen content. For example, photosynthetic capacity can decrease without a decrease in leaf nitrogen content in winter (Katahata et al. 2007), or with leaf age (Ishida et al. 1999; Escudero and Mediavilla 2003; Niinemets et al. 2004).

Photosynthetic capacity per unit leaf nitrogen (photosynthetic nitrogen-use efficiency; PNUE) is likely to be affected by climatic factors that change periodically. It has been shown, for example, that low temperature in winter (Miyazawa and Kikuzawa 2006; Zarter et al. 2006) or summer drought (Tenhunen et al. 1985; Wilson et al. 2000) leads to a reduction in photosynthetic capacity without a decrease in leaf nitrogen. In addition, growth irradiance may also be altered by development of overtopping new shoots and affect photosynthetic capacity of the leaves (Ishida et al. 1999; Niinemets et al. 2006).

Nitrogen partitioning within a leaf can also affect PNUE. PNUE decreases with increasing nitrogen partitioning to structural component at the expense of metabolic component (Onoda et al. 2004), and is sensitive to the balance between light-utilizing and light-capturing components in the photosynthetic apparatus, especially under changing growth irradiance (Hikosaka and Terashima 1995, 1996). How leaf nitrogen partitioning affects PNUE remains unclear in evergreens, partly because leaf nitrogen partitioning has only been studied during a limited period of the long leaf life, e.g. seasonal changes within a year (Miyazawa and Terashima 2001; Muller et al. 2005) and

age-related differences at a certain time of the year (Warren and Adams 2001; Miyazawa et al. 2004; Ethier et al. 2006; Katahata et al. 2007). Furthermore, many studies showed only indirect estimates of different nitrogen components, based on gas exchange parameters (e.g. Niinemets et al. 2004).

Leaf nitrogen partitioning is also important for understanding variation in nitrogen resorption, as different nitrogen components may be degraded and resorbed in different degrees (Kobe et al. 2005). In evergreen leaves, nitrogen resorption occurs while they are green as well as they are senescing (Millard and Proe 1992). It has been speculated that metabolic components are degraded and resorbed easily, while structural components are resistant to degradation and remain after resorption (Niinemets and Tamm 2005).

In this study, we examined temporal changes in leaf nitrogen partitioning in leaves expanded in outer and inner parts of the canopy of a broad-leaved evergreen, *Quercus myrsinaefolia*, growing in a temperate climate. Outer and inner leaves growing in different growth irradiance were compared as growth irradiance affects patterns of leaf nitrogen partitioning and various other leaf traits (Björkman 1981). We followed current-year and 1-year-old leaves for more than 1 year, which covered the leaf-life from developmental period to death in outer leaves. Leaf nitrogen partitioning was evaluated at two levels; between metabolic and structural proteins, and between chlorophyll and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) assuming that chlorophyll and Rubisco content represents the relative size of light-capturing and light-utilizing components of the photosynthetic apparatus. We addressed the questions: (1) are seasonal and yearly variations in PNUE related to leaf nitrogen partitioning? and (2) are different nitrogen components degraded in different degrees prior to nitrogen resorption?

Materials and methods

Study site and material plant

The study was conducted in the experiment garden of Forestry and Forest Products Research Institute (36°03′N, 140°10′E) from July 2006 through August 2007. The study site is characterized by a temperate climate, with the hottest and coldest month being August and January (Fig. 1a). The nearest weather station reported mean annual air temperature of 14.2 and 14.6°C and total precipitation of 1,616 and 1,136 mm in 2006 and 2007, respectively. There was no severe drought throughout the study period.

We selected five adult trees of *Quercus myrsinaefolia* (ca. 10 m) from a row of trees planted along a fence. The

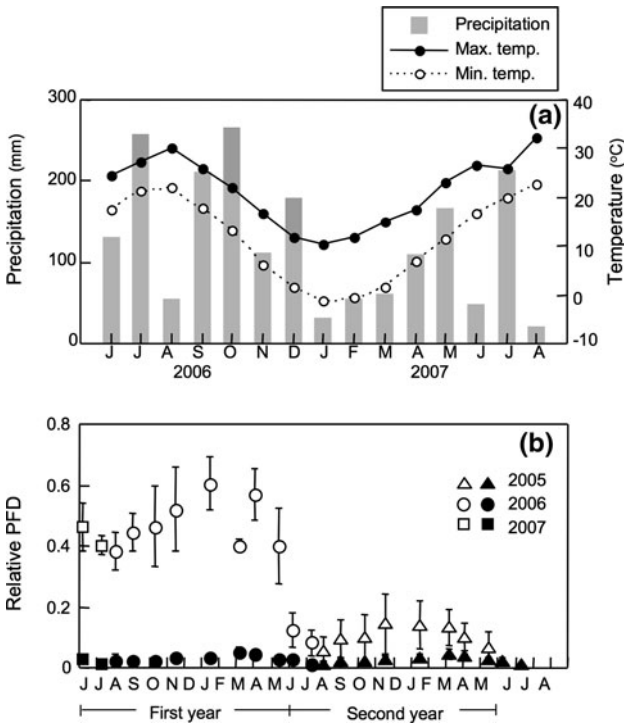

Fig. 1 Temporal changes in growth conditions. **a** Monthly total precipitation (*bars*), and average daily maximum (*closed circles*) and minimum temperature (*open circles*) provided by Japan Meteorological Agency. **b** Photon flux density relative to an open area under overcast skies. Leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in outer (*open symbols*) or inner (*closed symbols*) part of the crown. Values shown are the mean ± S.D.

trees developed new shoots in May–June, and abscised many old leaves around that time. Leaves formed in high-light conditions lived for around 2 years, but leaves formed in low-light conditions generally persisted longer, up to several years. We measured the amount of nitrogen compounds and photosynthetic capacity in leaves formed in 2005, 2006, and 2007, on a monthly interval, of outer shoots from fully-exposed south-facing branches and inner shoots that were deeply shaded inside the canopy. Leaf characteristics other than photosynthesis were determined also in "dead leaves", which were fully senescent and easily detached from the trees in June 2007. We measured the width and length of tagged leaves ($n = 15$, three leaves per tree) regularly and ensured that leaf area does not change after full expansion in the first June till death both in outer and inner leaves. Growth irradiance on outer and inner leaves ($n = 5$, one per tree) was measured as photon flux density relative to an open area with point sensors (LI-190SB, Li-Cor, Nebraska, USA) connected to a detalogger (MES-UL120, Koito, Tokyo, Japan) under overcast skies. PFD values were logged every 10 s for 3–5 min. Growth irradiance on outer leaves was fairly high in the first year of their life, but decreased considerably as new shoots

overtopped them in the beginning of the second year (Fig. 1b). Inner leaves were in shady conditions throughout their life.

Photosynthetic capacity

Light-saturated photosynthetic rates (P_{max}; $n = 10$, two leaves per tree) were measured during 8:30 am–12:30 pm in sunny days with a portable photosynthesis system (LI6400; Li-Cor, Nebraska, USA) under photon flux density of 1,500 μmol m^{-2} s^{-1}, CO_2 concentration of 370 μmol mol^{-1} and uncontrolled ambient temperatures. Stomatal conductance to water vapor (g_s), intercellular CO_2 concentration (C_i), leaf temperature and vapour pressure deficit (VPD) were recorded simultaneously. After P_{max} measurements, the leaves were sampled at the petiole and brought to the laboratory in damp plastic bags.

Leaf mass per area, chlorophyll content and nitrogen content

In the laboratory, leaf discs (diameter 8 mm) were punched out for biochemical analyses. One fresh disc was scraped and immersed in dimethylformamide, and the concentration of chlorophyll (Chl) extracted in it was determined with a spectrophotometer ($n = 10$; Porra et al. 1989). Several leaf discs were dried at 70°C for more than 72 h, weighed to determine leaf mass per leaf area (LMA; $n = 10$) and used to determine nitrogen concentration ($n = 10$) with an NC analyzer (Sumigraph NC-80, Sumika Chemical Analysis Service Ltd., Tokyo, Japan) connected to a gas chromatograph (GC-8A, Shimadzu Ltd., Kyoto, Japan). The extent of nitrogen resorption was calculated as:

$$\text{Nitrogen resorption efficiency } (\%) = (\text{pre-resorption } N_{area} - \text{post-resorption } N_{area})/\text{pre-resorption } N_{area} \times 100$$

where N_{area} is leaf nitrogen content per unit leaf area. The rest of the leaf discs were stored at −80°C until protein analysis.

Metabolic protein, structural protein and Rubisco

Leaf protein was divided into two fractions according to solubility to a detergent, sodium dodecyl sulfate (SDS). The SDS-soluble fraction is regarded as metabolic protein (the sum of soluble and membrane protein), and the SDS-insoluble fraction is regarded as structural protein associated with cell walls (Takashima et al. 2004). One or more frozen leaf discs ($n = 5$; one leaf per tree) were powdered in liquid nitrogen in a mortar with a pestle, and homogenized in 100 mM Bicine–NaOH buffer (pH 8.0) containing 20 mM $MgCl_2$, 10 mM dithiothreitol, 2% (w/v) SDS and 3% (w/v) polyvinylpolypyrrolidone (in case of metabolic protein) or polyvinylpyrrolidone (in case of structural protein). The homogenate was then centrifuged at 15,000 g for 20 min to recover metabolic protein in the supernatant and structural protein in the sediment. Metabolic protein was precipitated with trichloroacetic acid (final conc. 15% w/v), and structural protein was washed with ethanol. Proteins were hydrolyzed in an autoclave with 0.316 mmol $Ba(OH)_2$ and purified water (120°C, 0.12 MPa), and the amount of resulting amino acids was determined with ninhydrin method (McGrath 1972). Amino acids were first mixed with 1.32 M sodium acetate buffer containing 9.25% (v/v) glacial acetic acid and 0.1 mM NaCN, and then with 0.5% (w/v) ninhydrin in 2-methoxyethanol, and heated in the autoclave at 108°C for 5 min. The absorbance of developed color was read at 570 nm with the spectrometer. Protein standard was prepared with bovine serum albumin.

Rubisco was extracted from a frozen disc with the Bicine-NaOH buffer as described for metabolic protein, and quantified with SDS–polyacrylamide gel electrophoresis (Laemmli 1970). The bands of Rubisco large subunit were cut out from the gel stained with Coomassie Brilliant Blue R-250, eluted in formamide at 50°C for 5 h, and the absorbance was read at 595 nm with the spectrophotometer (Makino et al. 1986). Rubisco purified from *Spinacia oleracea* was used as a standard.

Statistical analyses

Statistical tests were performed with StatView software version 5.0 (SAS Institute, Inc., Cary, NC, USA). Regression lines were obtained using the least-squares methods.

Results

Leaf nitrogen content (N_{area}) increased from the first summer towards winter in outer leaves (Fig. 2a). Inner leaves also showed a moderate increase in N_{area} during the same period. N_{area} decreased from the winter towards spring, due to nitrogen resorption prior to new shoot development. Nitrogen resorption occurred twice during the life of outer leaves; once while leaves were green (the period from the first winter to the second spring) and once while leaves were senescing (the period from the second winter to the third spring). The efficiency of nitrogen resorption was significantly higher in outer leaves than in inner leaves while they were green ($P < 0.05$, Table 1), but the lifetime nitrogen resorption efficiency was similar between outer and inner leaves ($P = 0.16$).

LMA was relatively stable, but showed a marked reduction from the first winter towards the second spring especially in outer leaves (Fig. 2b). LMA showed a slight tendency to increase with age in inner leaves, but not in outer leaves. There was no reduction in LMA associated with leaf senescence prior to death.

Light-saturated photosynthetic rate (P_{max}) and stomatal conductance (g_s) changed similarly during the life of outer and inner leaves (Fig. 3a, b). Intercellular CO_2 concentration (C_i) showed a moderate variation. In outer leaves, P_{max} decreased from the peak in the first summer towards the first winter, and decreased further to the following spring. P_{max} was retained at a low level during the second year and dropped sharply just before the leaf death in the third spring. In inner leaves, the temporal variation was smaller, and P_{max} did not decrease in autumn either in the first and the second year. They, however, showed a similar decline in P_{max} from winter to spring.

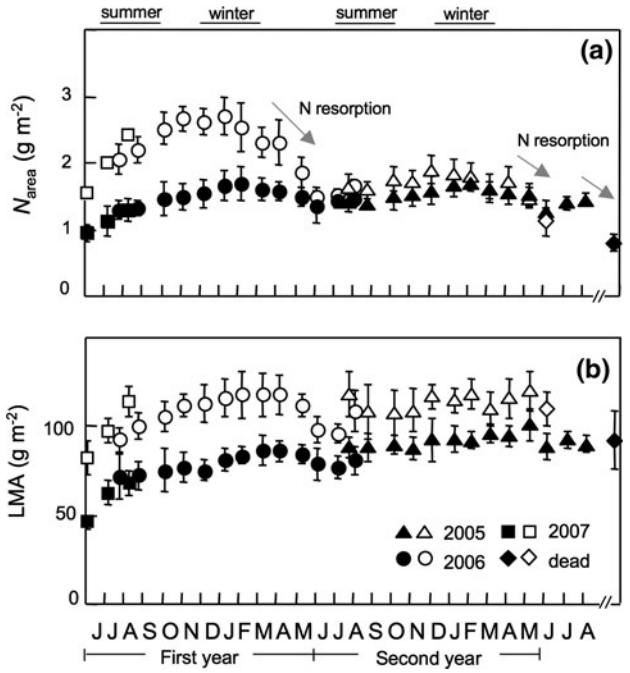

Fig. 2 Temporal changes in **a** leaf nitrogen content (N_{area}) and **b** leaf mass per leaf area (*LMA*) of leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in either outer (*open symbols*) or inner (*closed symbols*) part of the crown. Dead leaves are shown as *diamonds*. Values shown are the mean ± S.D.

P_{max} and N_{area} were only weakly correlated with each other through leaf life (Fig. 4). In outer leaves, PNUE (P_{max} per N_{area}; equivalent to the slope of the line from the origin to each point in Fig. 4) was low during leaf expansion, reached the peak in the first summer, decreased towards the first winter and further towards the second year. Inner leaves showed similar but much less pronounced temporal changes in PNUE.

Metabolic protein and structural protein explain 70–80% and ca. 10% of N_{area} in the first summer, respectively (we assumed that nitrogen concentration of the proteins to be 16%). Metabolic protein and structural protein content changed differently with seasons (Fig. 5). Metabolic protein content decreased at the time of nitrogen resorption (from winter to spring) and became lowest when leaves were dead. Structural protein content was relatively stable throughout the leaf life span, and did not decrease even during leaf senescence.

Rubisco content changed drastically during leaf life span, while Chl content showed a moderate variation (Fig. 6). Rubisco and Chl were degraded to a large extend during leaf senescence.

Both metabolic protein and Rubisco content showed close correlations with N_{area} in outer and inner leaves (Fig. 7a, c). Structural protein content correlated with LMA (Fig. 7b, inset), but not with N_{area} (Fig. 7b). Chl content was not as closely correlated with N_{area} (Fig. 7d) as Rubisco. Outer leaves showed smaller Chl per N_{area} than inner leaves in the first year, but Chl per N_{area} increased moderately in the second year.

Discussion

Seasonal variation during the first year of leaf life

During the first year, N_{area} and P_{max} changed greatly among seasons in outer leaves (Figs. 2a, 3a). The temperature seemed to have placed a major impact on P_{max}, as its values increased from spring towards summer and decreased from autumn towards winter, together with

Table 1 Nitrogen resorption efficiency (R_{EFF}) of outer and inner leaves of *Q. myrsinaefolia*

	Green-leaf R_{EFF} (%)	Senescing-leaf R_{EFF} (%)	Lifetime R_{EFF} (%)
Outer leaves	45 ± 5.3	40 ± 14.7	58 ± 7.9
Inner leaves	20 ± 14.2	53 ± 7.7*	52 ± 12.6

Green-leaf R_{EFF}, Senescing-leaf R_{EFF}, Lifetime R_{EFF} denote the % reduction in nitrogen content from the its peak in the first summer till the second spring, from its peak in the second summer till leaf death, and from its peak in the first summer till leaf death, respectively. The mean ± S.D.

* Calculated as the % reduction from the second summer till leaf death

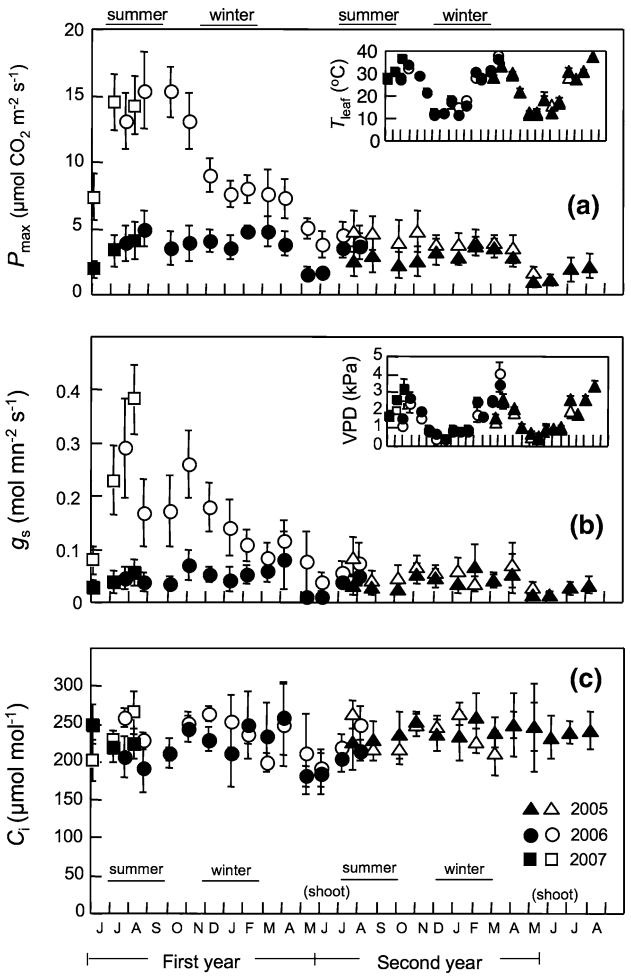

Fig. 3 Temporal changes in **a** light-saturated photosynthetic rates (P_{max}), **b** stomatal conductance to water vapor (g_s), and **c** intercellular CO_2 concentration (C_i) of leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in either outer (*open symbols*) or inner (*closed symbols*) part of the crown. *Inset* in **a** and **b** shows leaf temperature (T_{leaf}) and vapor pressure deficit (*VPD*) at the time of photosynthetic measurements, respectively. Values shown are the mean ± S.D.

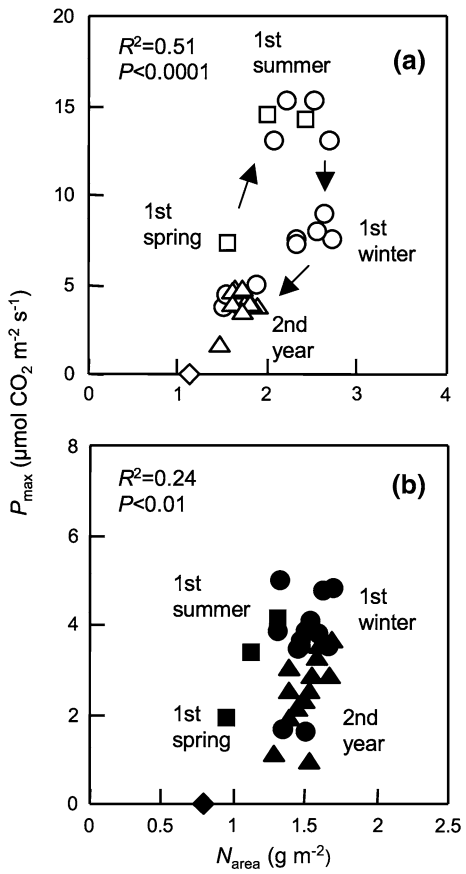

Fig. 4 Relationships between light-saturated photosynthetic rates (P_{max}) and nitrogen content (N_{area}) in leaves expanded in **a** outer or **b** inner part of the crown. *Triangles*, *circles* and *squares* denote leaves produced in 2005, 2006 and 2007, respectively, and *diamonds* denote dead leaves. Each point indicates the mean of one sampling date

temperature. By faded color of the sun-lit surface we speculate that outer leaves suffered from photoinhibition associated with low temperature and high light in winter, the phenomenon common in temperate forests (Katahata et al. 2005; Miyazawa et al. 2007). Inner leaves, by contrast, showed no such reduction in P_{max}.

As P_{max} and N_{area} changed differently, PNUE showed a large seasonal variation especially in outer leaves (Fig. 4). In the first spring PNUE was especially low, probably because nitrogen was preferably partitioned towards structural proteins at the time of leaf development and metabolic proteins were synthesized later (Fig. 5). Other studies have also shown that the photosynthetic apparatus develops slowly after leaf emergence in evergreens (Miyazawa and Terashima 2001; Miyazawa et al. 2003). The sharp drop in PNUE from the summer towards winter was not associated with the change in leaf nitrogen partitioning to metabolic protein and Rubisco, which increased with N_{area}, or structural protein that did not change in amount (Fig. 7a, b, c). Why should leaves invest such a large amount of metabolic protein in winter, when photosynthesis is likely to be depressed by low temperature? This large amount of metabolic protein may partially compensate for the reduction in specific activity of enzymes in winter. Alternatively, leaves may only be storing excess nitrogen in the form that is easy to degrade and remobilize in the following spring. It has been suggested that some evergreens store nitrogen in the form of Rubisco under suboptimal conditions (Warren and Adams 2004), although we found that total amount of metabolic protein, but not only Rubisco, increased linearly with N_{area} in *Q. myrsinaefolia* (Fig. 7a, c). PNUE decreased further from winter to the following spring. This might have been caused by increased energy consumption (respiration) for protein degradation and nitrogen resorption (Collier and

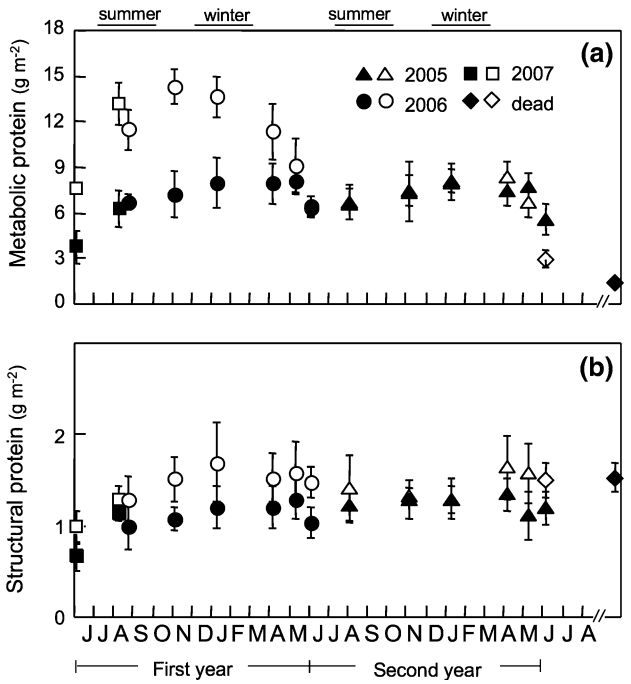

Fig. 5 Temporal changes in **a** metabolic protein content and **b** structural protein content of leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in either outer (*open symbols*) or inner (*closed symbols*) part of the crown. Dead leaves are shown as *diamonds*. Values shown are the mean ± S.D.

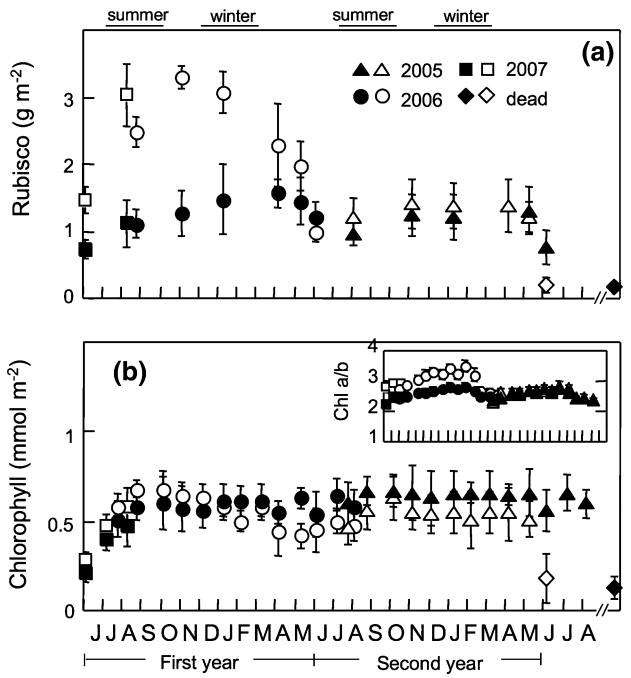

Fig. 6 Temporal changes in **a** Rubisco and **b** chlorophyll content and chlorophyll a/b ratio (*inset*) of leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in either outer (*open symbols*) or inner (*closed symbols*) part of the crown. Dead leaves are shown as *diamonds*. Values shown are the mean ± S.D.

Thibodeau 1995), but PNUE did not recover in the second year after the nitrogen resorption period.

Yearly variation between the first and second year of the leaf life

Outer leaves experienced a drastic change in growth irradiance when they were shaded by new shoots in the second spring (Fig. 1b). In response to shading, outer leaves showed physiological changes common to many species (e.g. Brooks et al. 1994; Eichelmann et al. 2005) such as increases in Chl per N_{area} (Fig. 7d) and decreases in Chl a/b (Fig. 6b, inset). Increases in nitrogen investment towards light-capturing components can enhance photosynthesis under shady conditions (Hikosaka and Terashima 1995). LMA did not decrease with shading (Fig. 2b) as morphological traits are generally unchangeable after full expansion (Yamashita et al. 2002; Oguchi et al. 2005).

PNUE halved in the second year from its value in the first year, not only in outer leaves but also in inner leaves that were under consistent growth irradiance (Fig. 4). Thus, PNUE decreased with leaf age in *Q. myrsinaefolia*. Age-related decline in PNUE has been observed in other evergreen species as well (Escudero and Mediavilla 2003; Niinemets et al. 2004). We found no such drastic decreases in leaf nitrogen partitioning to metabolic protein (Fig. 7a) or increases in nitrogen partitioning to structural protein (Fig. 7b) in the second year that can explain the age-related decline in PNUE in *Q. myrsinaefolia*. Nitrogen partitioning within the photosynthetic components, between Rubisco and Chl, was also relatively stable in inner leaves (Fig. 7c, d). Taken together, our results suggest that leaf nitrogen partitioning is not a major factor driving the age-related decline in PNUE. Other physiological factors, such as the activation state of Rubisco and regulation of electron transport, should be more important in explaining the effect of ageing on photosynthesis (Ethier et al. 2006).

Nitrogen resorption from overwintering leaves in spring

Nitrogen resorption is an important conservation mechanism that allows plants to recycle this growth-limiting nutrient internally (Aerts and Chapin 2000). In evergreens, nitrogen resorbed from overwintering leaves supports new shoot growth independently from current nitrogen uptake from the soil (Millard and Proe 1992; Millard et al. 2001; Cherbuy et al. 2001). In *Q. myrsinaefolia*, the extent of nitrogen resorption varied within the same crown (Table 1). Outer leaves exhibited higher nitrogen resorption efficiency than inner leaves while they were green, probably because demand from new shoots (Miyazawa et al. 2004), and/or maximum leaf nitrogen content before resorption (Fife et al. 2008) affects the amount of nitrogen

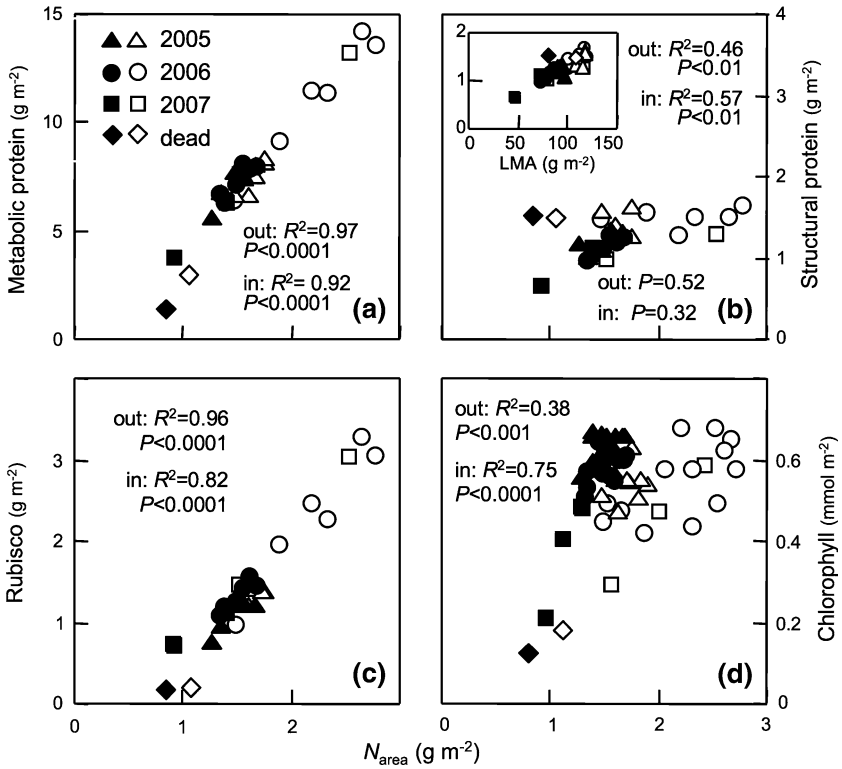

Fig. 7 **a** Metabolic protein, **b** structural protein, **c** Rubisco and **d** chlorophyll content as a function of leaf nitrogen content (N_{area}) in leaves expanded in 2005 (*triangles*), 2006 (*circles*) and 2007 (*squares*) in either outer (*open symbols*) or inner (*closed symbols*) part of the crown. Dead leaves are shown as *diamonds*. *Inset* in **b** shows relationships between structural protein content and leaf mass per area (*LMA*). Each point indicates the mean of one sampling date

being resorbed. Nitrogen resorption during leaf senescence was more efficient and thorough than when leaves were green, and left only a small amount of nitrogen in dead leaves (Fig. 2a). Nitrogen resorption from senescing leaves, which is likely to be unrelated to sink size (Miyazawa et al. 2004), may always be maximized to reduce nitrogen loss from the plant.

Metabolic protein could explain the bulk of nitrogen being resorbed in *Q. myrsiaefolia* (Fig. 7a), as have been observed in a herb *Chenopodium album* (Yasumura et al. 2007) and a deciduous species *Lindera umbellata* (Yasumura et al. 2006). Regardless of growth forms, metabolic protein seems to be degraded and resorbed to a large extent. In contrast, structural protein was small in quantity and made a negligible contribution to nitrogen resorption in *Q. myrsiaefolia* (Fig. 7b). Degradability of structural protein seems to vary among species, from highly degradable (up to 90% in *C. album*), moderate (30% in *L. umbellata*) to resistant (10% in *Q. myrsinaefolia*). Such an interspecific difference may be ascribed to leaf structural properties that vary depending on leaf habits and life forms (Garnier and Laurent 1994; Castro-Diéz et al. 2000). In *Q. myrsinaefolia*, metabolic and structural protein together explained around 60% of nitrogen remaining in dead leaves. Therefore, the process of protein degradation was partially limiting the extent of nitrogen resorption during leaf senescence.

Researchers have speculated that thylakoid proteins are more difficult to degrade and resorb than soluble proteins in stroma (Aerts 1996). Our results showed that chlorophyll-proteins do not always contribute to nitrogen resorption as readily as Rubisco, if adjustment to growth irradiance is underway at the same time (Fig. 7c, d). In dead leaves, however, there was only a negligible amount of Chl left (Fig. 6b), indicating that chlorophyll-proteins could greatly contribute to nitrogen resorption during leaf senescence. Similarly, both the soluble and chlorophyll-protein (referred to as "membrane protein" therein) were degraded to a large extent in senescing leaves of a herb *C. album* (Yasumura et al. 2007).

Conclusion

In *Quercus myrsinaefolia* leaves, metabolic protein content changed in harmony with total leaf nitrogen content. Structural protein content was relatively stable after the completion of full leaf expansion until death. Nitrogen partitioning between Rubisco and chlorophyll was affected by growth irradiance as well as total leaf nitrogen content. Seasonal and yearly variation in PNUE could not be explained completely by leaf nitrogen partitioning; environmental factors such as temperature and internal factors other than nitrogen partitioning seem to contribute to the

temporal variation in PNUE. During nitrogen resorption, metabolic protein served as the main source of nitrogen. Rubisco was degraded and resorbed more readily than chlorophyll-proteins, but both were degraded almost thoroughly during leaf senescence. Structural protein had low degradability and comprised non-retranslocatable nitrogen pool in the leaf.

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